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PRINCIPAL INVESTIGATOR: Nina Schor, M.D., Ph.D.

CONTRACTING ORGANIZATION: Children's Hospital of Pittsburgh  
Pittsburgh, Pennsylvania 15213

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*Mina F. Schor* 5/19/98  
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#### (4) TABLE OF CONTENTS

	Page
Front Cover	1
Report Documentation Page (SF 298)	2
Foreword	3
Table of Contents	4
Introduction	5
Body	7
Experimental Methods, Assumptions, and Procedures	7
Results and Discussion	8
Conclusions	10
References	11
Appendices (includes Figures 1-3)	14

## (5) INTRODUCTION

The proto-oncogene *bcl-2* is expressed in 65-80% of human breast cancers (2,18). Approximately 50% of breast cancers are found to overexpress this gene (1-2). Overexpression of *bcl-2*, which results in overproduction of the 26 kD protein Bcl-2, has been shown to confer resistance to apoptotic cell death induced by a variety of stimuli, including chemotherapeutic-induced apoptosis in breast cancer (3-4). This is particularly important in the case of advanced, estrogen receptor-positive tumors, a circumstance in which Bcl-2 content correlates with clinical resistance to chemotherapy (4).

Recent studies in other cell lines have suggested that Bcl-2 exerts its protective effects by a mechanism that includes a shift in the redox potential of the cell to a more reduced state (5,16). Among other biochemical effects, these studies specifically implicate an increase in cellular reduced glutathione (GSH) content in this alteration in redox potential. This makes particular sense because other studies link the apoptotic process to exposure to reactive oxygen species (19-21). One potential strategy for overcoming Bcl-2-mediated chemotherapeutic resistance is to take advantage of the increase in cellular GSH and, therefore, free sulfhydryl content by using chemotherapeutic agents that require reduction by sulfhydryl compounds for their activity. We have recently explored *in vitro* the efficacy of one such group of agents, the enediynes, in pheochromocytoma cells that were genetically engineered to overexpress *bcl-2* (11).

Neocarzinostatin (NCS) is an enediyne DNA-cleaving natural product that induces apoptosis in some tumor cell lines in culture (9). Like other naturally-occurring enediynes, NCS is actually a prodrug that requires sulfhydryl activation for efficacy. As such, the cytotoxicity of NCS has been demonstrated to vary directly with the sulfhydryl content of the cell (7, 14-15). This information led to our prediction that, contrary to the case for all other chemotherapeutic agents studied, overexpression of *bcl-2* and the resulting shift in redox potential of the cell would potentiate the induction of apoptosis by NCS.

We have shown that, in PC12 rat pheochromocytoma cells that have been *bcl-2*- or control-transfected, *bcl-2* overexpression does indeed potentiate the apoptosis-inducing activity of NCS by increasing cellular levels of GSH (11). The reduction-dependent enediyne prodrugs are therefore the only class of drug that has been demonstrated to work best in those tumor cells that have become resistant to other known chemotherapeutic agents.

Although the naturally-occurring enediynes have been difficult to implement clinically because of toxicity issues (22), recent advances in pharmacology and pharmaceuticals have begun to get around these problems. Modifying the NCS chromophore structure to produce other more efficacious and/or less toxic enediynes (23), masking the protein component with inert polymers (24), using enediynes adjunctively with cell-selective activating agents (7), and coupling the newer enediynes to monoclonal antibodies directed at tumor-specific antigens (25) have all proved useful in producing enediyne candidates for human therapeutics. In fact, the last of these approaches is now in Phase I/II clinical trials for ovarian carcinoma (L. Hinman, personal communication), and has shown promise in a breast cancer cell line in culture (25). The 4-fold gap in sensitivity to NCS that we observed between *bcl-2*- and control-transfected PC12 cells (11) has led us to speculate that the enediynes would be both more efficacious and safer in these patients than in those whose tumors do not overexpress *bcl-2*.

These interesting and promising preliminary results led us to predict that breast cancer cells that overexpress *bcl-2* would have a higher GSH content and therefore be more susceptible to the effects of the enediynes than are breast cancer cells that do not

overexpress this gene or normal cells. We further predicted that we could augment the difference in enediyne sensitivity between *bcl-2*-native normal and -overexpressing tumor cells by increasing the availability of cysteine, the rate-limiting substrate for GSH synthesis. Furthermore, we predicted that by increasing the Bcl-2 content of estrogen receptor-positive breast cancer cells with tamoxifen (17) or estrogen (3), we could increase the effectiveness of enediynes against these cells, raising the possibility of adjunctive treatment with enediynes and tamoxifen. This would translate clinically into an improved therapeutic index for treatment of breast cancer with the enediynes.

## (6) BODY

### (6)-1. Experimental Methods, Assumptions, and Procedures

All of the methods we proposed for use in these studies are currently established in our laboratory using cultured cell systems (8-12, 27-32). We describe below the specific hypotheses that were tested and the methods that were used in this first year of Department of Army funding.

#### (6)-1.1: General Methods Applicable to All Tasks and Technical Objectives

MCF-7 (estrogen receptor-positive) cells were obtained from the American Type Culture Collection (Rockville, MD), and were maintained as stock cultures in  $\alpha$ -MEM (GIBCO-BRL) containing phenol red and supplemented with nonessential amino acids, 0.3% glucose, 5% fetal bovine serum, and 2  $\mu$ g/ml gentamicin sulfate (37°C, 5% CO<sub>2</sub>). Where indicated, studies were conducted under four sets of conditions known to alter *bcl-2* expression in this cell line in predictable ways: maintenance of the conditions of the stock cultures (E+; *bcl-2*-positive at "resting" levels; 3-4); addition to the stock culture conditions of 1 nM 17 $\beta$ -estradiol and maintenance in the estradiol-enriched medium for 48 hr prior to study (E++; 3-fold enhancement of *bcl-2* content over resting levels; 4); maintenance for 7 days prior to study in phenol red-free DMEM (GIBCO-BRL) containing 5% fetal bovine serum stripped of steroids by absorption to dextran-coated charcoal (Sigma Chemical Corp., St. Louis, MO) for 45 min at 45°C (E-; 6-fold reduction of *bcl-2* levels relative to resting state; 3). In addition, E+ cells were studied after a four-day exposure to tamoxifen (10<sup>-6</sup>M; condition T+), a condition the *in vivo* analogue of which is associated with induction of *bcl-2* (17). In all cases, the same lot of fetal bovine serum (GIBCO-BRL) was used for all conditions in each experiment, and to the extent possible, for all experiments.

In addition, clonal transfectants of MCF-7 human breast cancer cells were obtained from Drs. Charles Rudin and Craig Thompson (University of Chicago, Chicago, IL). These transfectants were produced by electroporation (300 mV, 960 mFD) with pSFFV-neo and pSFFV-*bcl-2* (plasmids described in ref. 38), respectively.

(6)-1.2: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

For all studies, relative *bcl-2* content of native MCF-7 cells maintained under each condition was assayed by Western blotting as we have previously described (11). In the case of MCF-7 transfectants, stably transfected clones were screened for Bcl-2 production by Western blot analysis using the N-19 anti-Bcl-2 antibody (Santa Cruz Biotechnology).

(6)-1.3: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

To determine the GSH and total glutathione (GSH+GSSG) contents of MCF-7 cells containing different amounts of *bcl-2*, E+, E++, E-, and T+ cells (10<sup>7</sup> of each) were washed free of medium, and suspended in 1 ml of phosphate-buffered saline (PBS). The suspension was homogenized and assayed for GSSG and GSH+GSSG by the method of Tietze (33). The rate of change in the OD<sub>412</sub> was measured spectrophotometrically over a period of 3 min. This rate was converted to the total glutathione concentration by plotting on a simultaneously run standard curve for  $\Delta$ OD<sub>412</sub> versus total glutathione concentration (constructed using GSH standard solutions). The concentration of GSSG alone was determined by an identical procedure performed on samples that were treated with N-

ethylmaleimide prior to assay, to eliminate the reaction of GSH with DTNB (33). The cellular contents of GSH and GSH+GSSG were then calculated for each condition. Each determination was performed in triplicate. Values so obtained for each experiment were compared between E+ and E++, E-, or T+ cells using Student's t test, as we have done in our published work (27-28).

(6)-1.4: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

An NCS concentration-response study was conducted for E+, E++, E-, and T+ MCF-7 cells and MCF-7 transfectants, as we have previously described for pheochromocytoma cells (11). Cells plated in 6-well tissue culture plates were treated with a range of concentrations of NCS (0-0.5  $\mu$ g/ml) for 1 hr at 37°C. Cultures were then washed free of NCS, and adherent cell number was determined daily in control- and NCS-treated E+, E++, E-, and T+, and MCF-7 transfectant cultures as we have previously described for neuroblastoma and pheochromocytoma cells (6,8,11). The statistical significance of differences between E+ and E++, E-, and T+ cells, in turn, was assessed for each concentration of NCS using Student's t test, with  $p \leq 0.05$  being considered significant.

(6)-1.5: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript co-authored by Drs. Schor, Kagan and his laboratory colleagues, and Thompson and his laboratory colleagues is in the final stages of preparation. The BRCA IDEA award from the Department of the Army is acknowledged in this manuscript.

## **(6)-2. Results and Discussion**

(6)-2.1: Task 1 [Verification by Western blotting of effects of manipulations of estrogen exposure (i.e., conditions E++, E+, E-, T+) on *bcl-2* content of MCF-7 cells]

We have measured the effects of addition to and subtraction from the medium of estradiol to the Bcl-2 content of MCF-7 cells in culture. Our first experiments involved using complete medium as the control, and adding various amounts of estradiol to the medium, as originally described in the proposed Methods. These studies, all using the same batch of serum for constitution of the medium, demonstrated no change in the Bcl-2 content of the cells. Our assumption was that the serum-replete medium contained sufficient estradiol that neither our addition of small amounts of this compound nor our partial depletion of estradiol with charcoal treatment made a significant change in the Bcl-2 content. We surmounted this problem in two ways. First, we obtained estrogen-free medium commercially stripped (by column chromatography) of estrogen, and performed our studies by adding back various amounts of estradiol. This study conclusively demonstrated the estrogen concentration-dependent increase in concentration of Bcl-2 in the cells (See Figure 1). In addition, we have obtained from the laboratory of Dr. Craig Thompson (University of Chicago, Chicago, IL) two clones of MCF-7 cells that have been transfected with the *bcl-2* gene, and their mock-transfected counterparts. We have confirmed over-production of Bcl-2 in the *bcl-2*-transfected cells, and have maintained all of these lines in our laboratory.

(6)-1.2: Task 2 [Measurement of concentrations of GSH, GSSG, and total glutathione (GSH+GSSG) in E++, E+, E-, and T+ MCF-7 cells]

We have now measured the total glutathione content of the transfectants obtained from Dr. Thompson's laboratory. Accompanying the increase in Bcl-2 content afforded by the *bcl-2* transfection, there was a small but significant increase in glutathione content (see



Figure 2). In contrast, and in accordance with the demonstration of no change in Bcl-2 content, results from a single experiment recently completed demonstrate that, in native MCF-7 cells maintained in complete medium with added estradiol, there is a paradoxical small and not statistically significant decrease in the glutathione content with no change in the ratio of reduced to oxidized species. These studies suggest a relationship between Bcl-2 content and total glutathione content of MCF-7 cells.

(6)-1.3: Task 3 [Performance of concentration-response studies for E++, E+, E-, and T+ MCF-7 cells and MCF-7 transfectants exposed to neocarzinostatin]

Our previous studies have demonstrated the paradoxical increase in sensitivity of PC12 rat pheochromocytoma cells to NCS afforded by *bcl-2* transfection (11). This potentiation of NCS toxicity was abrogated by prior incubation of PC12 cells with BSO. To determine the generalizability of these findings to the overexpression of *bcl-2* in different biological systems, similarly transfected MCF-7 human breast cancer cells were examined for the effects of *bcl-2* overexpression on sensitivity to NCS. Unlike PC12 cells, *bcl-2* overexpression protected MCF-7 cells from death induced by NCS (Figure 3). At each concentration, the survival of the *bcl-2* transfectants exceeds that of the mock transfectants.

Attempts to perform the same set of studies in cells exposed to E++, E+, E-, and T+ or E- and graded addition of E conditions were thwarted by the tendency of estrogen-deprived cells to lift off of the culture surface over the final 5-10 days of the experimental exposure.

(6)-1.4: Task 4 (Preparation of a manuscript describing the findings relative to Tasks 1-4)

A manuscript to be submitted for publication is currently in draft form. This manuscript is entitled "Glutathione handling in *bcl-2*-transfected tumor cell lines: Implications for chemotherapy", and is co-authored by NF Schor, C Rudin, A-R Hartmann, CB Thompson, Y Tyurina, and VE Kagan. The support of the Department of the Army Breast Cancer Program has been acknowledged in this manuscript.

## (7) CONCLUSIONS

The studies performed to date indicate that, at least in cultured breast cancer cell lines, increased sensitivity to NCS is not an inevitable consequence of altered Bcl-2 content. This is in contradistinction to the human pheochromocytoma cells on which we have previously reported (11). We have recently obtained preliminary results in connection with another project focussed on neuroblastoma and funded by the NIH, that indicate that this is because the relationship between Bcl-2 content and altered handling of GSH is cell line-dependent. We plan, in the next several months, to test this hypothesis in our human breast cancer model. This is in direct concert with the proposed studies for months 13-24 in our original IDEA Award application. These studies of the effects of Bcl-2 content on GSH handling and chemosensitivity may be of broad applicability, not only to breast cancer, but to the fundamental mechanism of action of Bcl-2 in the tumor cell.

Our studies also indicate that changes in Bcl-2 content require extreme changes in estrogen exposure (from estrogen deprivation to estrogen supplementation), and that these conditions may not be realistic reflections of the environment in which most breast cancers sit *in vivo*. The fact that serum in medium contains sufficient estrogen to maximally augment Bcl-2 content in MCF-7 cells is a reflection of this conclusion.

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## (9) APPENDICES

Figure 1: Western blot for Bcl-2 of MCF-7 cells treated with varying concentrations of estradiol and PC12 cells transfected with the bcl-2 gene.

Figure 2: Total glutathione +/- SEM of mock- and bcl-2-transfected MCF-7 cells.

Figure 3: NCS concentration-response curves for MCF-7 transfectants.

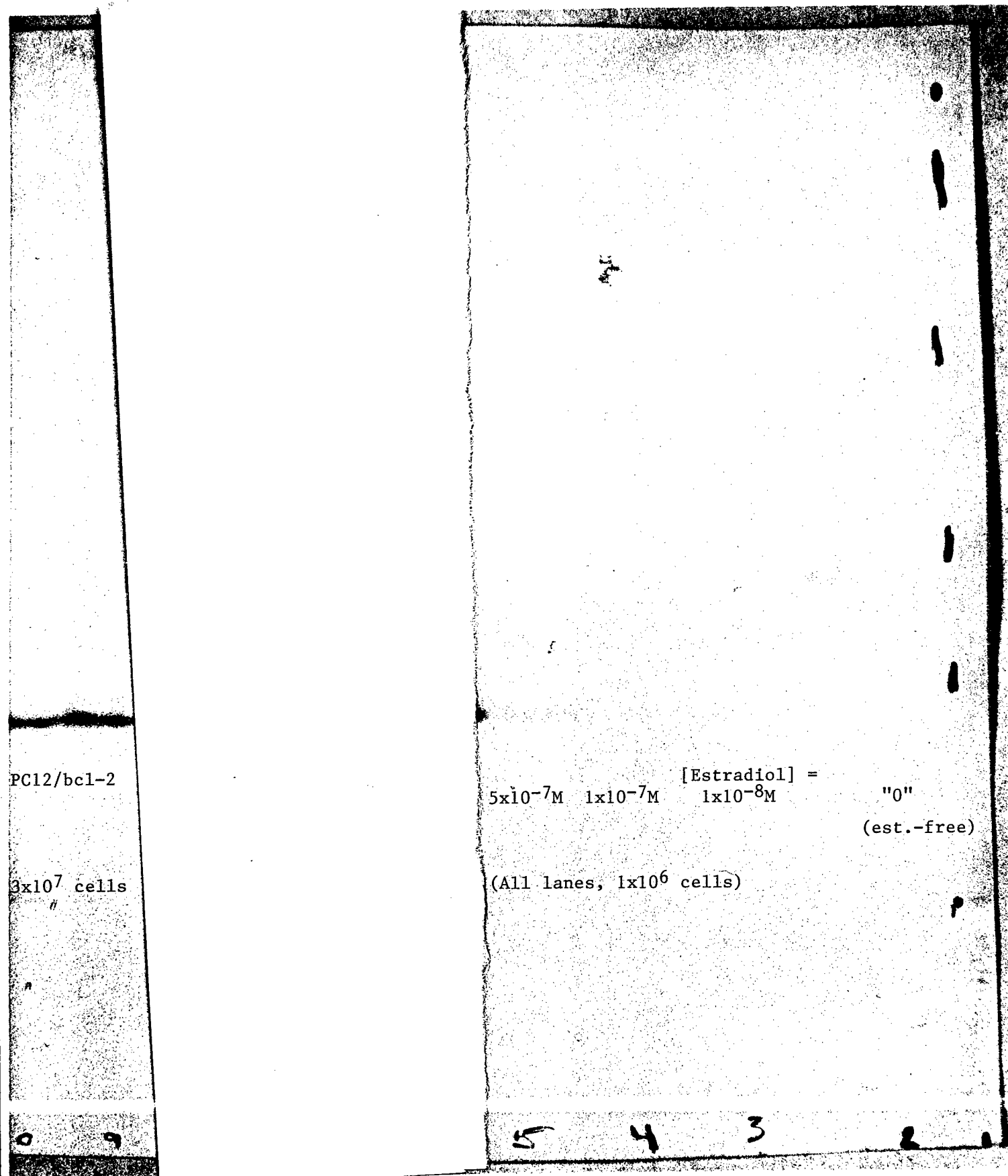


FIGURE 1: Western Blot for Bcl-2 of MCF-7 Cells Treated with Varying Concentrations of Estradiol (lanes 2-5) and PC12 Cells Transfected with the bcl-2 Gene (lane 9)

Figure 2: Total Glutathione +/- SEM of Mock- and bcl-2-transfected MCF-7 Cells

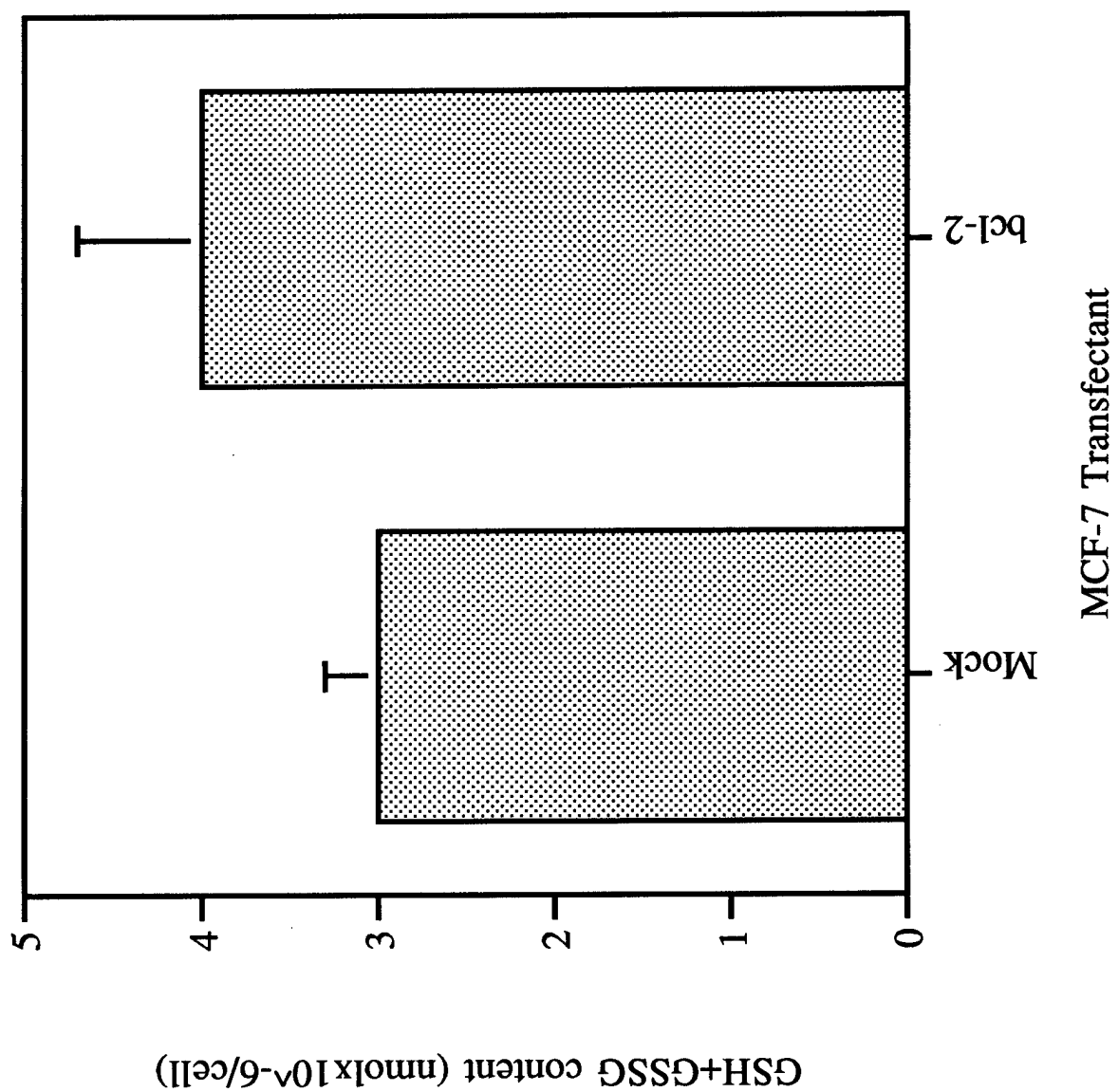




Figure 3: NCS Concentration-response Curves for MCF-7 Transfectants

